Základní metody molekulární genetiky

Petra Pokorná

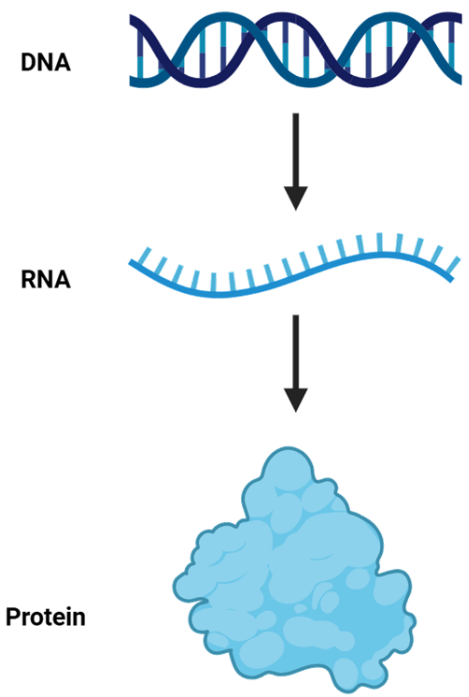
Obsah přednášky

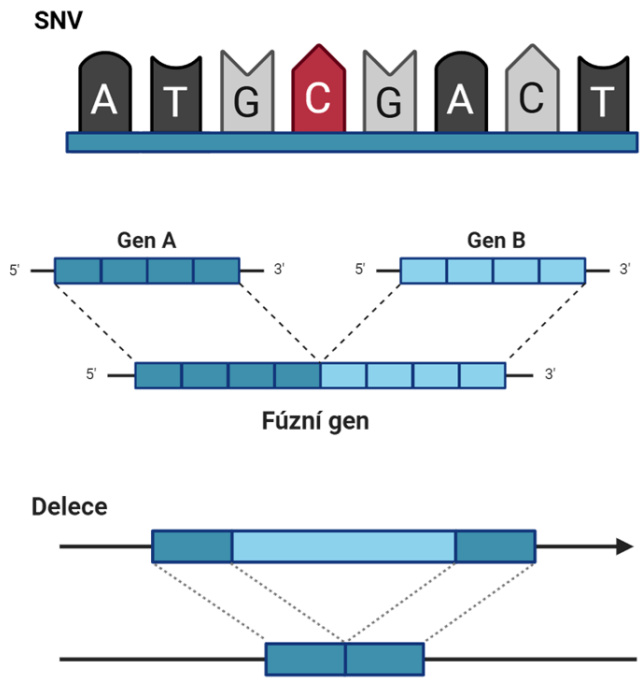
Imunohistochemie (IHC)

Fluorescenční *in situ* hybridizace (FISH)

Polymerázová řetězová reakce (PCR)

Sekvenování nové generace (NGS)

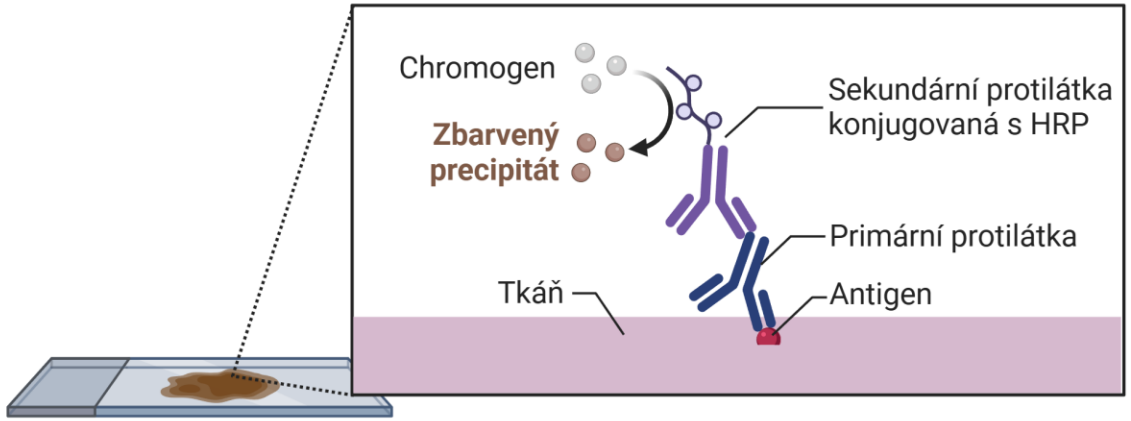




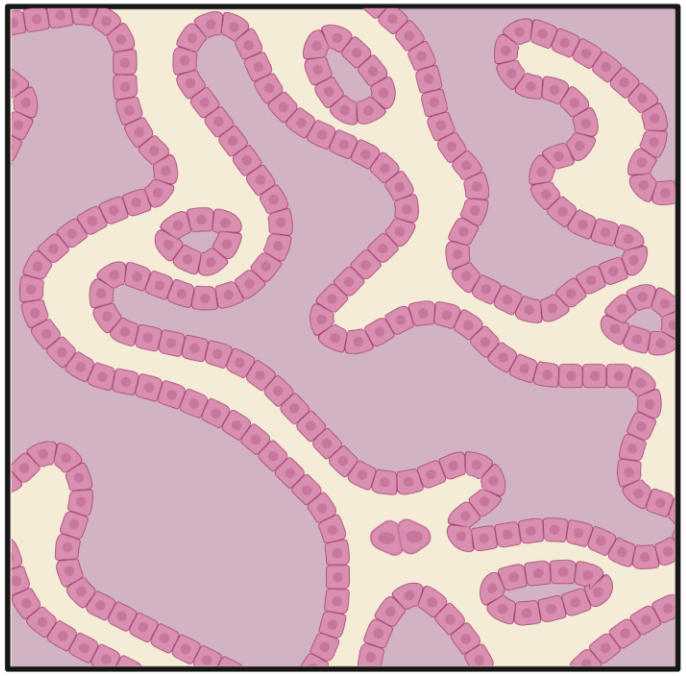
https://app.biorender.com/

Imunohistochemie

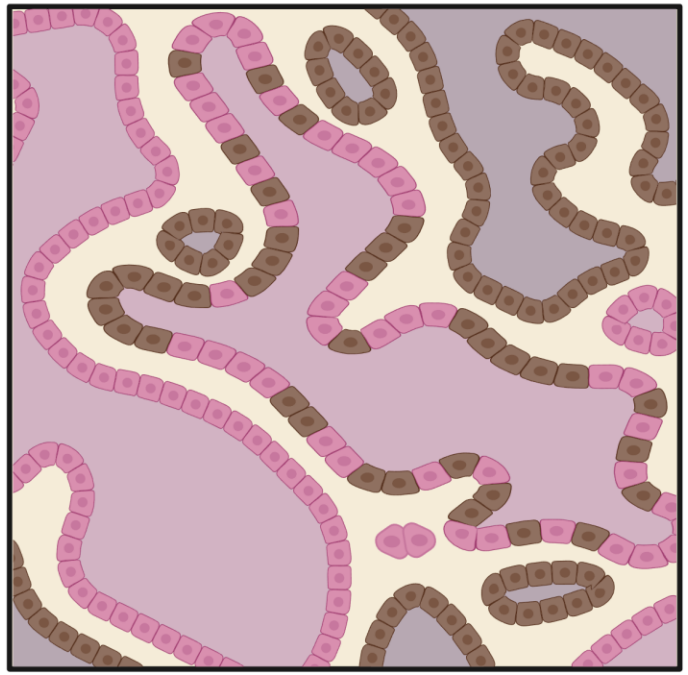
- Průkaz proteinů v nádorové tkáni
- Vazba specifické protilátky
- Hodnocení intenzity/rozsahu zabarvení



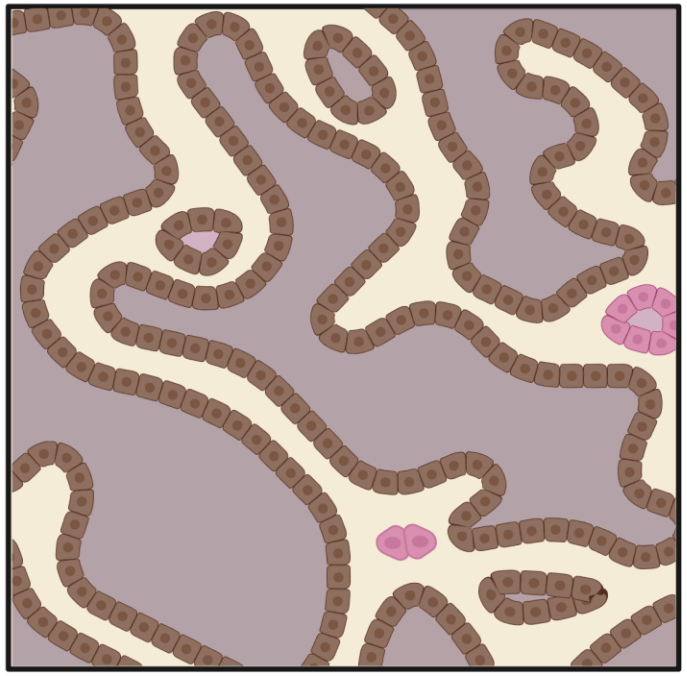
Negativní



Slabě pozitivní



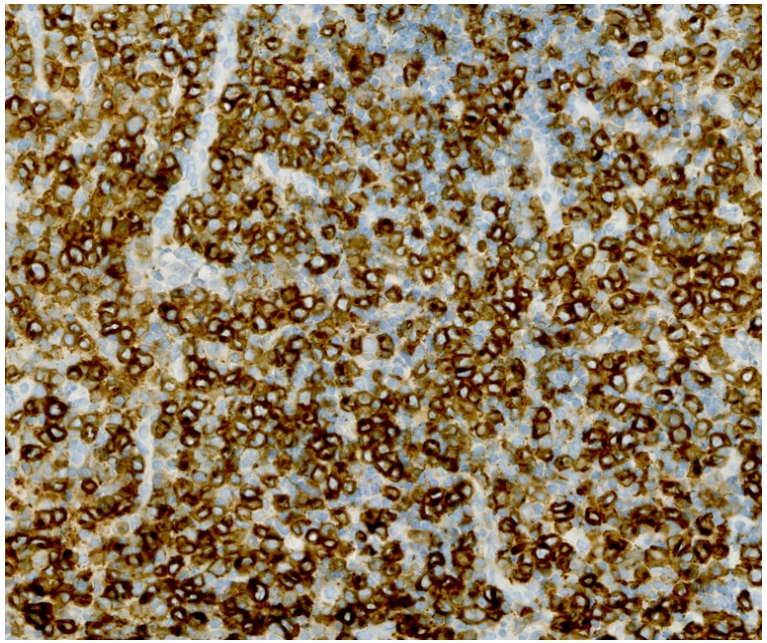
Pozitivní



https://app.biorender.com/

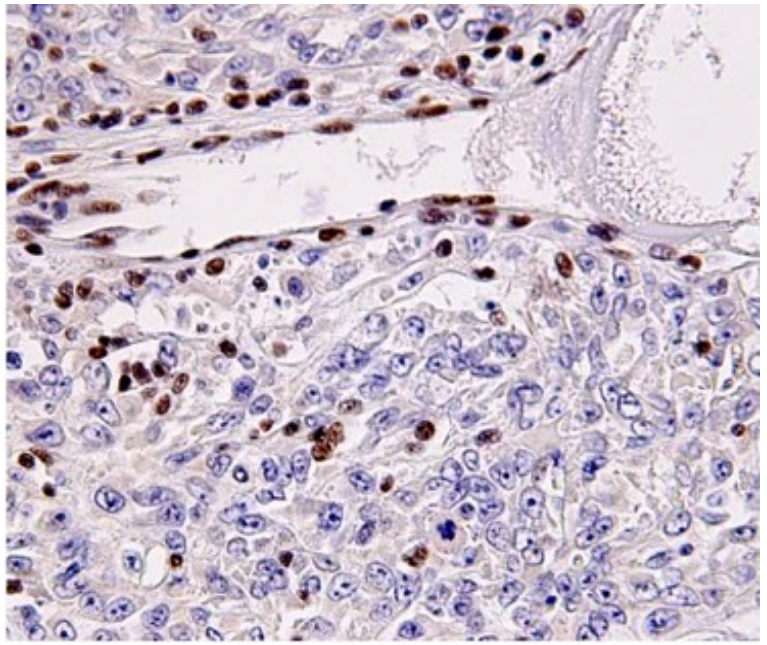
Wild-type protein

- Exprese specifických markerů (PD-L1)
- Zvýšená exprese proteinu v důsledku amplifikace/translokace (ALK, HER2 aj.)



Exprese ALK u *NPM1::ALK* fúzovaného ALCL

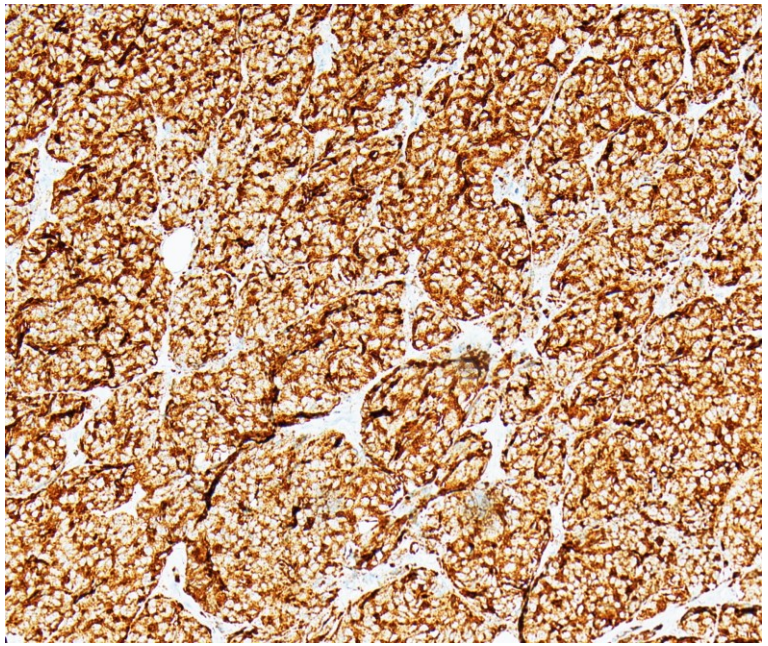
- Ztráta exprese svědčící pro inaktivační alteraci (SMARCB1, ATRX aj.)
- Ztráta exprese MMR proteinů – indikátor MSI



Ztráta exprese SMARCB1 u AT/RT

Mutovaný protein

- Přítomnost specifické mutace (BRAF V600E, IDH1 R132H aj.)

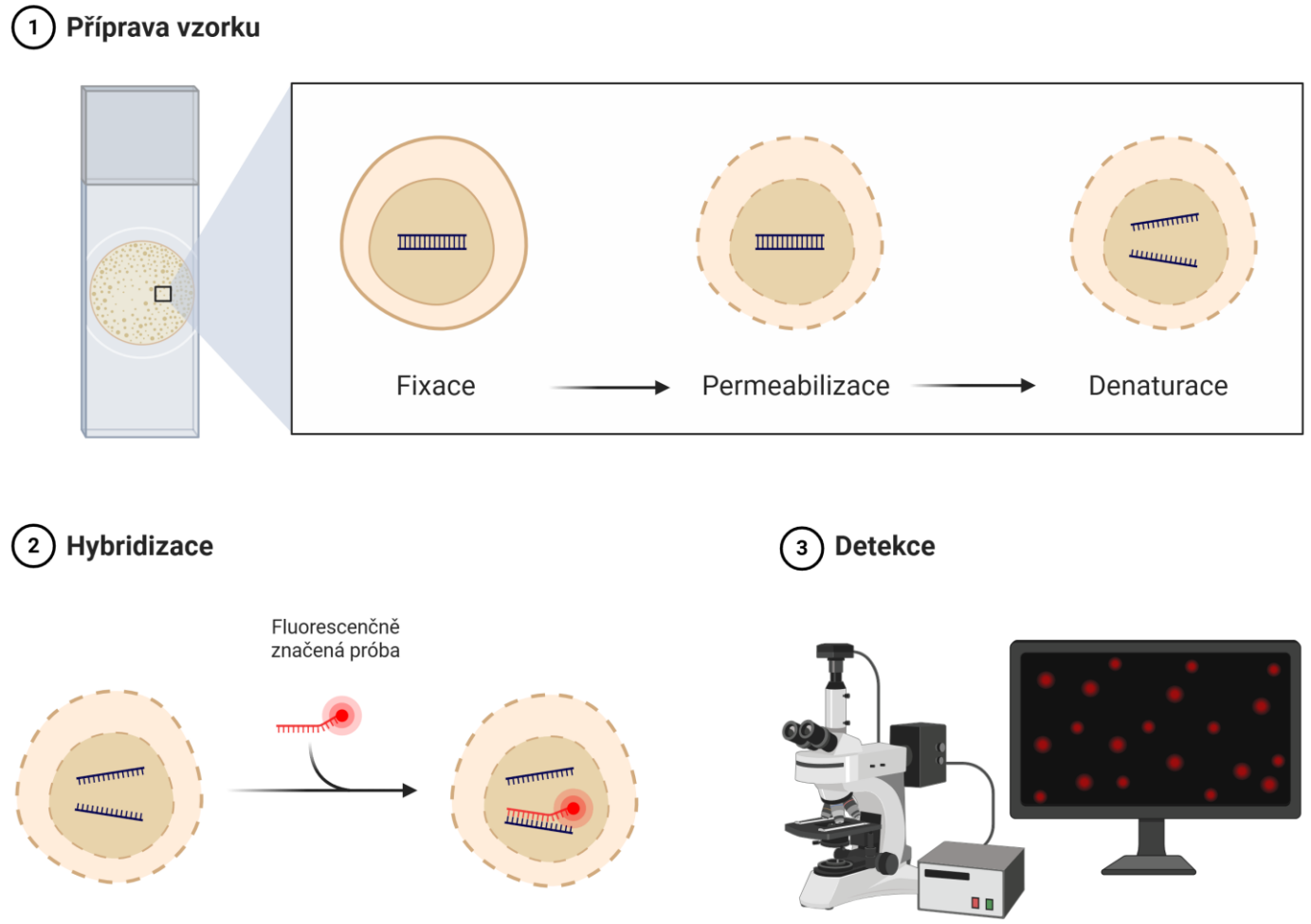


BRAF V600E u maligního melanomu

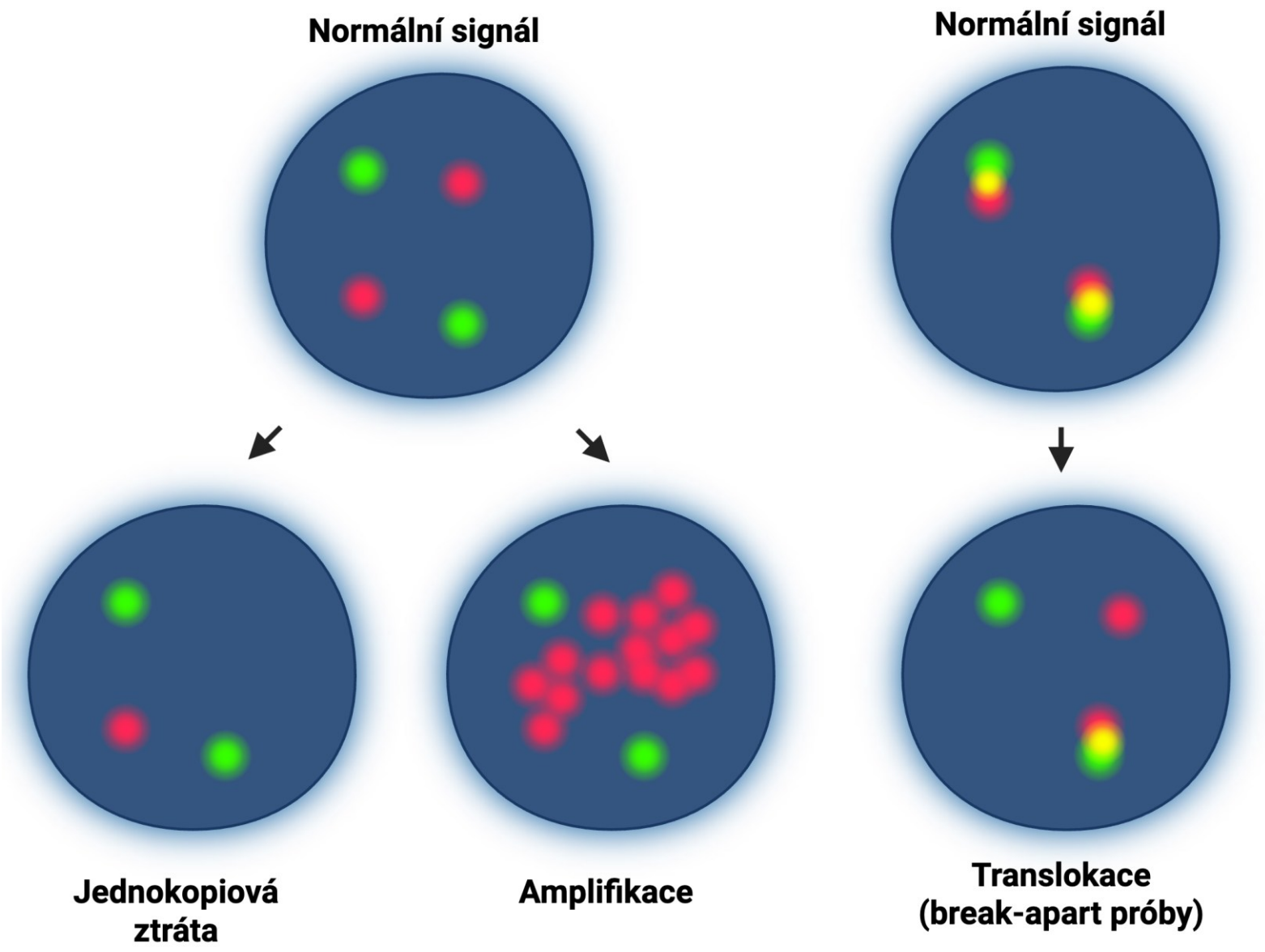
https://www.pathologyoutlines.com, PMID: 16528370

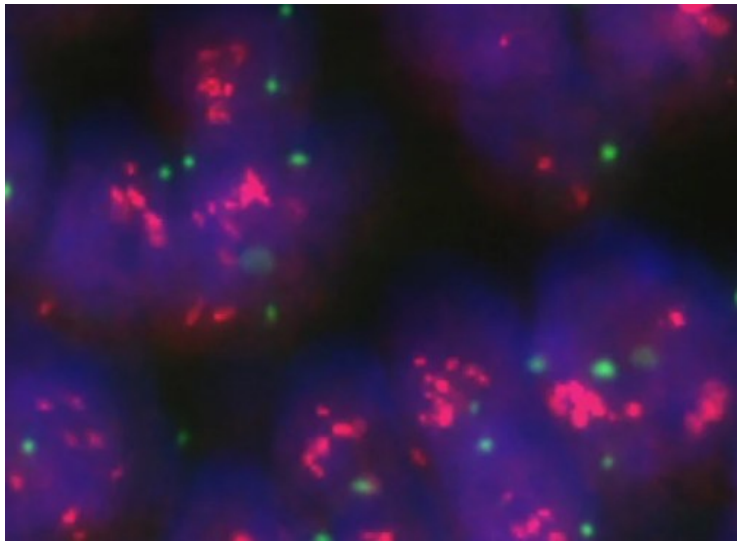
Fluorescenční *in situ* hybridizace

- Detekce na úrovni DNA v nádorové buňce pomocí fluorescenčně značených sond
- Počet signálů a jejich poměr ke kontrolnímu signálu, jejich uspořádání
- Změny počtu kopií, translokace

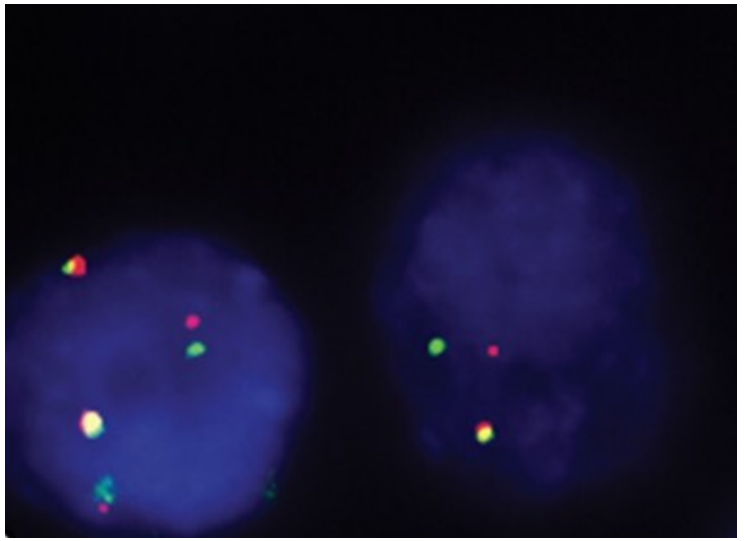


https://app.biorender.com/





HER2 amplifikace

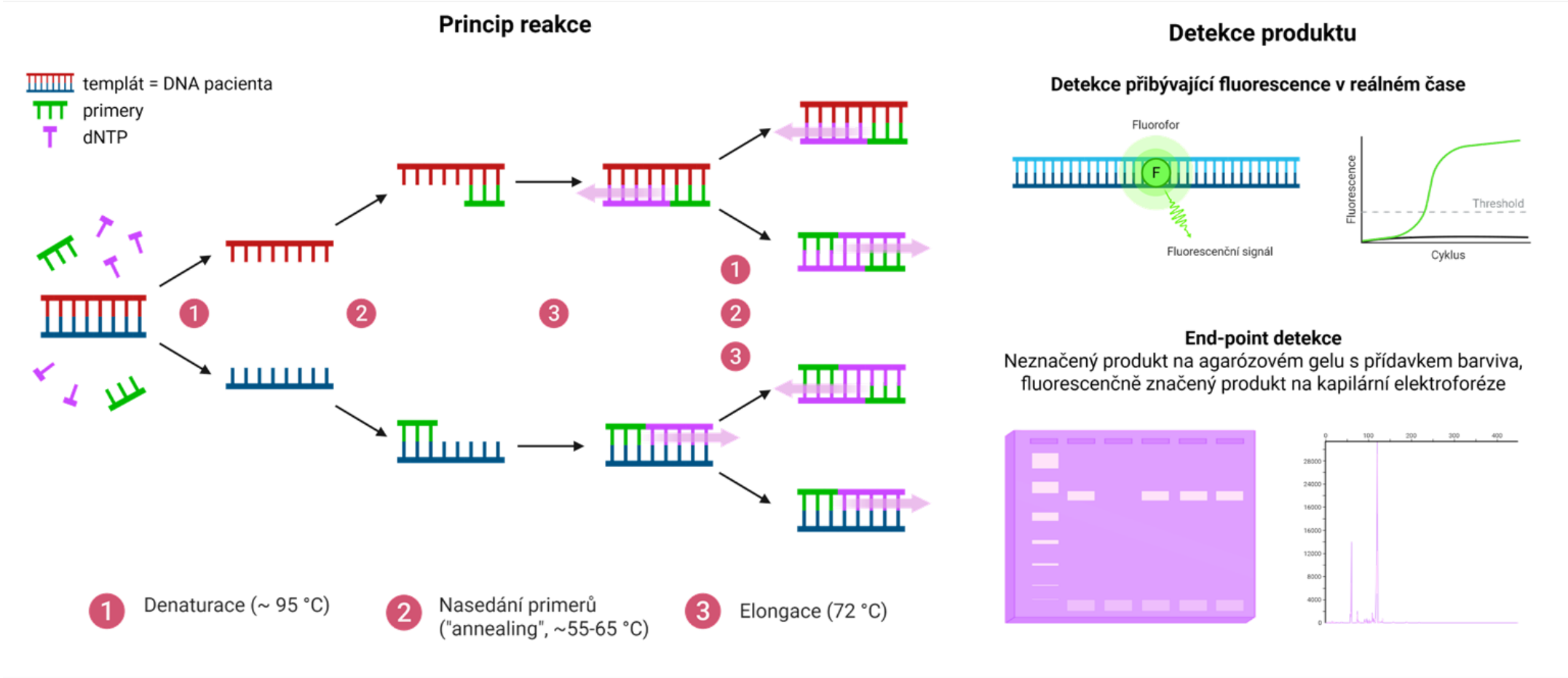


RET přestavba

https://aglient.com/

Polymerázová řetězová reakce

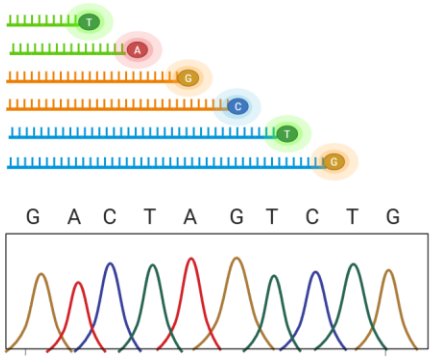
- Na rozdíl od předchozích metod nutná izolace NK z primárního materiálu
- Detekce na úrovni DNA (analýza RNA po předchozím přepisu do cDNA = tzv. RT-PCR)
- Analýza známých alterací (mutace, fúze)



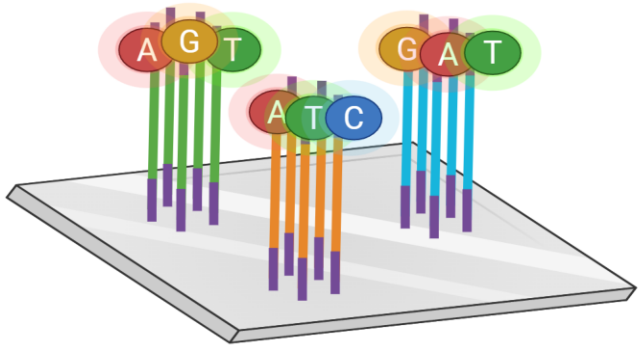
https://app.biorender.com/

Sekvenování

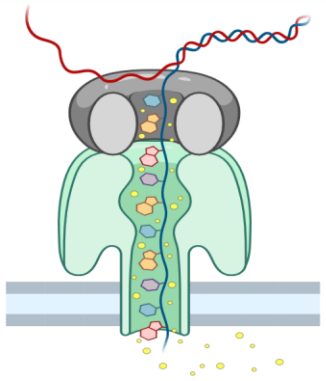
- Stanovení primární struktury DNA (analýza RNA po předchozím přepisu do cDNA)
- **1. generace** = Sangerovo sekvenování
  - cílená mutační analýza konkrétních úseků DNA
- **2. generace** = sekvenování nové generace (NGS) = masivně paralelní sekvenování
  - vysokokapacitní profilování, v současné praxi nejvíce využíváno
- **3. generace**
  - čtení dlouhých úseků bez nutnosti amplifikace



1. generace

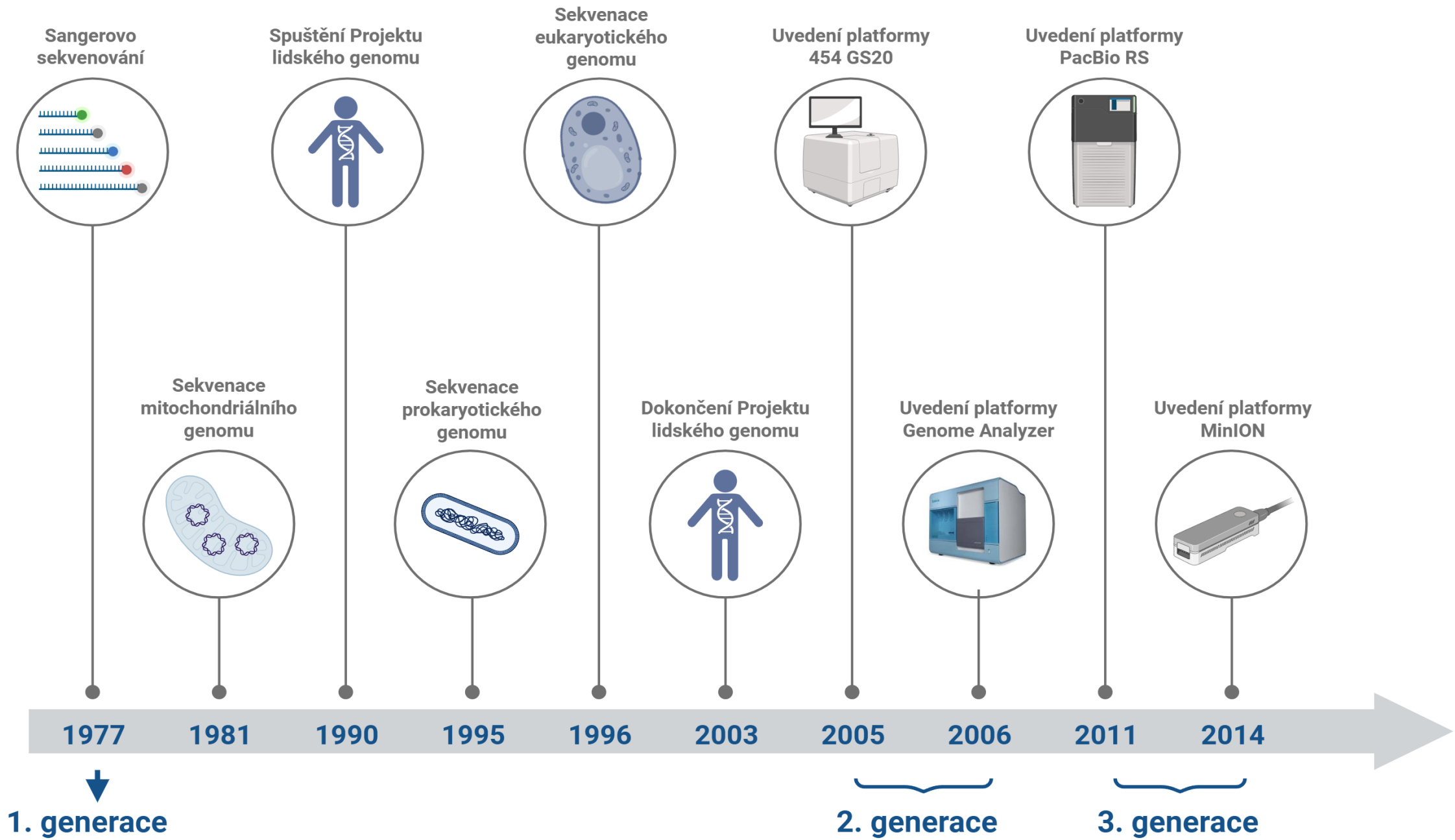


2. generace



3. generace

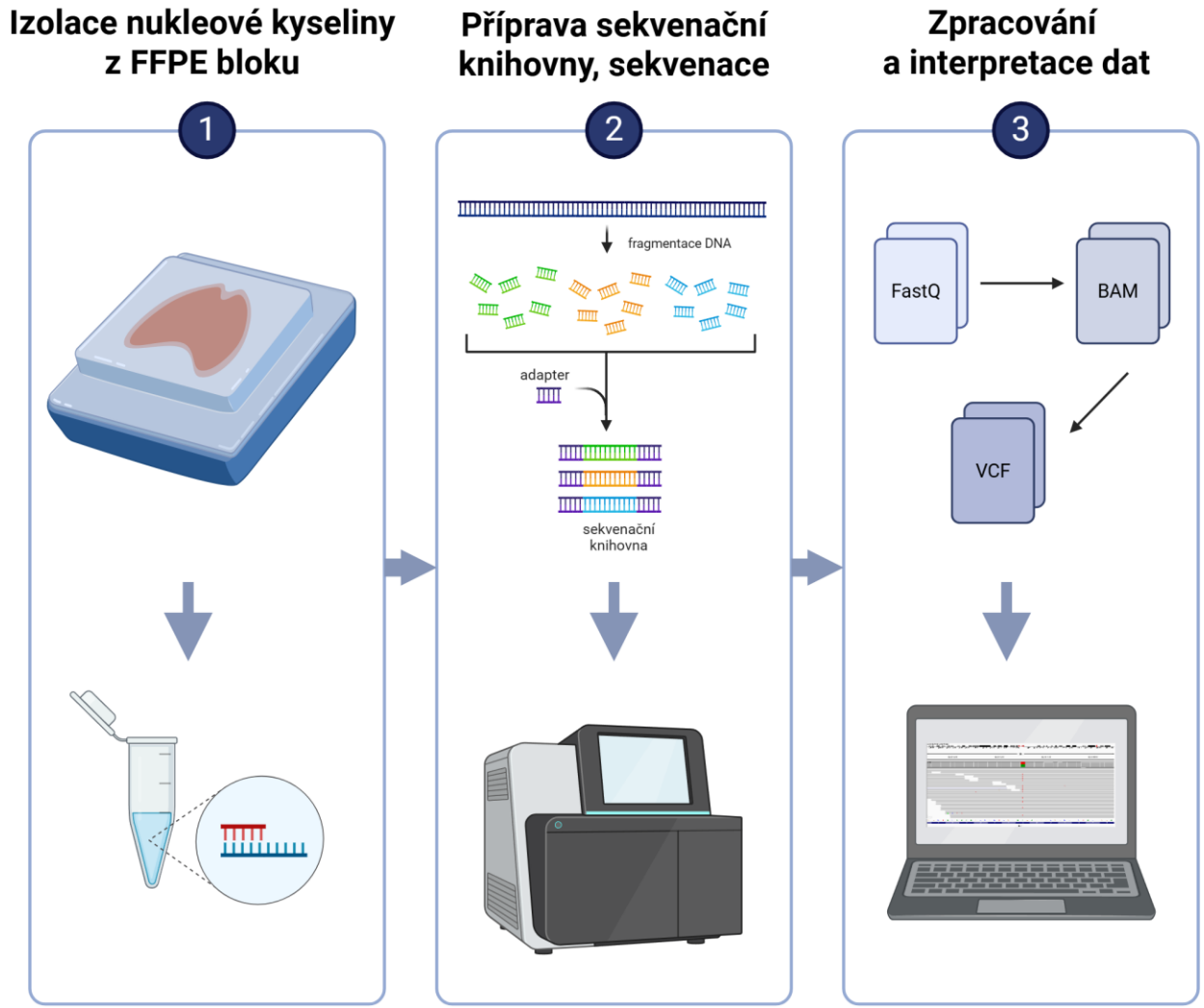
https://app.biorender.com/



https://app.biorender.com/

Sekvenování nové generace

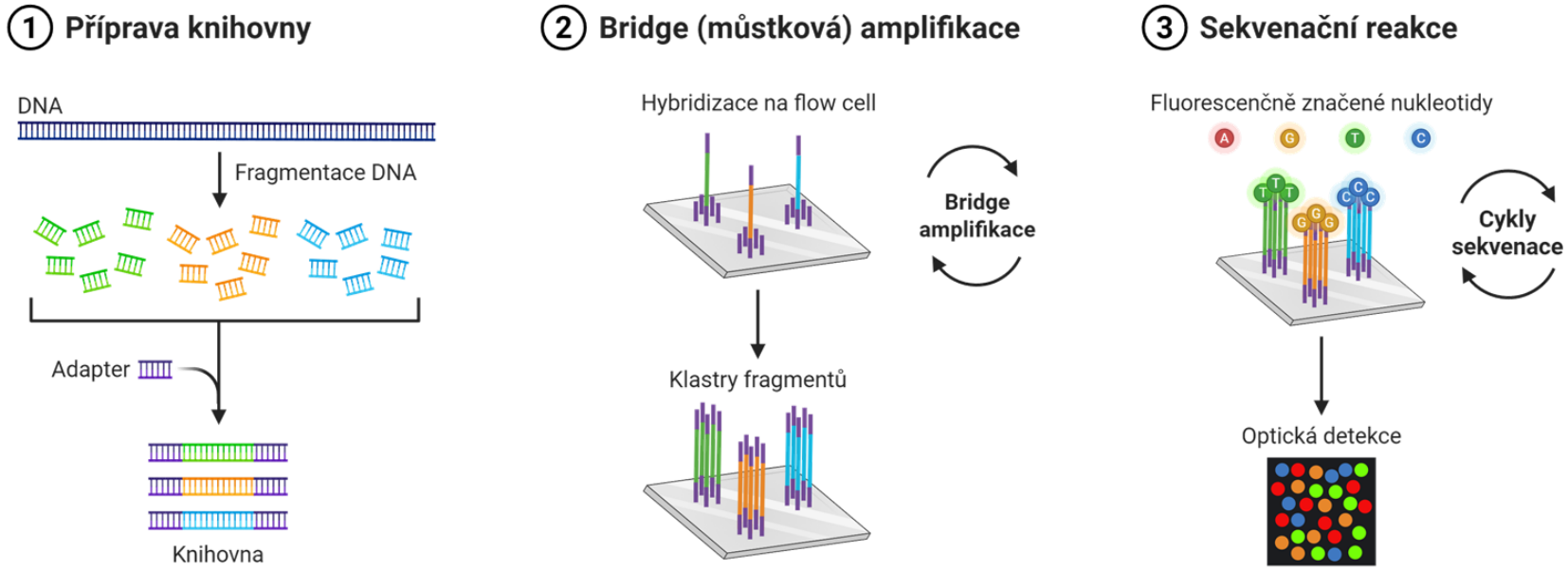
- Několikakrokový proces
  - Izolace DNA/RNA
  - Příprava sekvenační knihovny (knihovna = uskupení všech sekvenovaných fragmentů)
  - Sekvenace
  - Bioinformatické zpracování dat
  - Vyhodnocení – filtrování na základě různých kritérií, práce s databázemi, verifikace výsledků



https://app.biorender.com/

Sekvenování nové generace

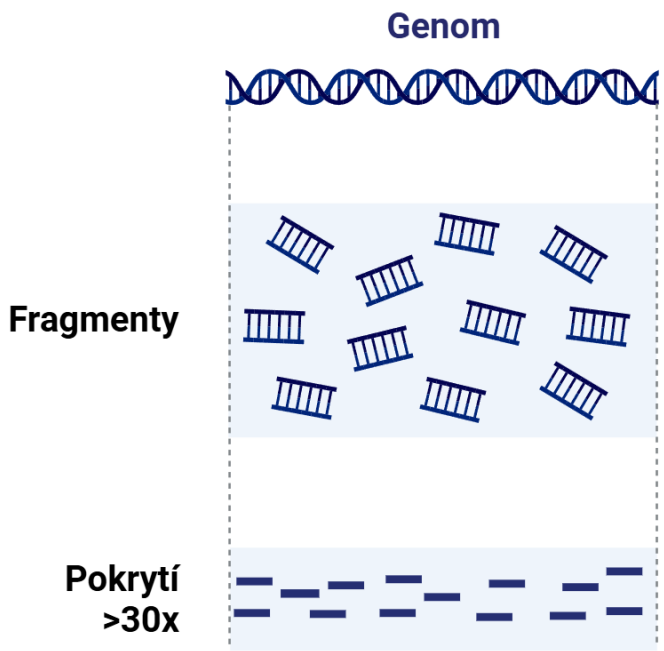
- Několik platforem sekvenování nové generace – nejčastější jsou však platformy od firmy Illumina charakteristické tzv. sekvenací syntézou
- Fragmenty knihovny jsou pomocí adapterových sekvencí přichyceny na sekvenační povrch (flow cell), namnoženy (bridge amplifikace) a následně probíhá samotná sekvenace syntézou = postupně je syntetizován komplementární řetězec, přičemž začlenění každého jednoho nukleotidu vede k emisi světla, které detekuje optický modul sekvenátoru
- https://youtu.be/fCd6B5HRaZ8



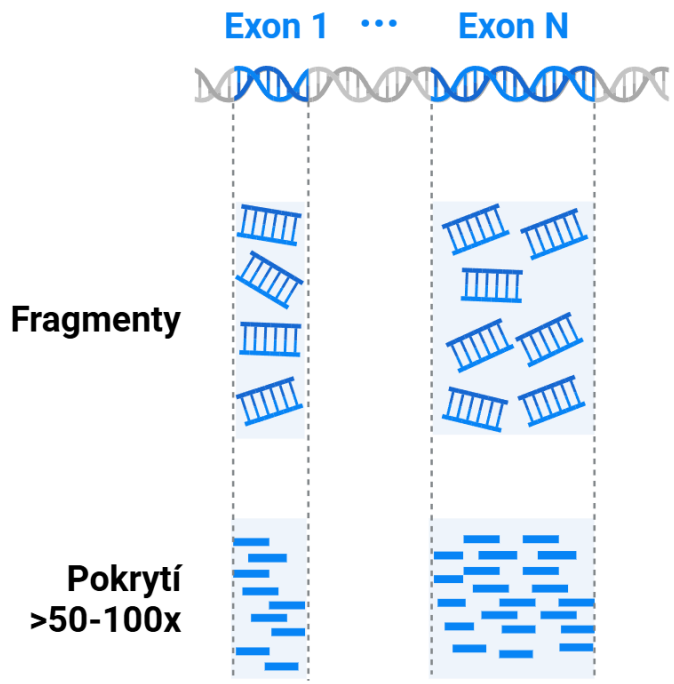
https://app.biorender.com/

Sekvenování nové generace

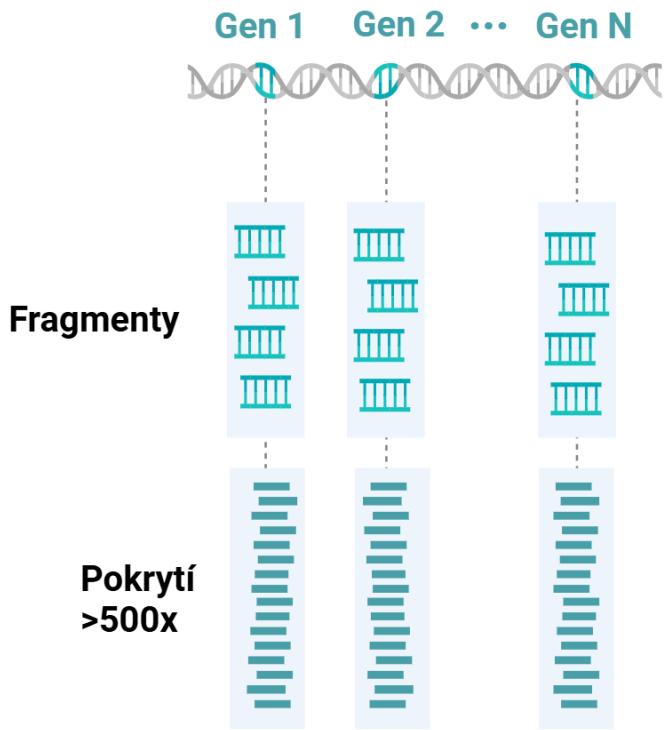
Celogenomové



Celoexomové



Cílené/panelové



CGP NGS panely

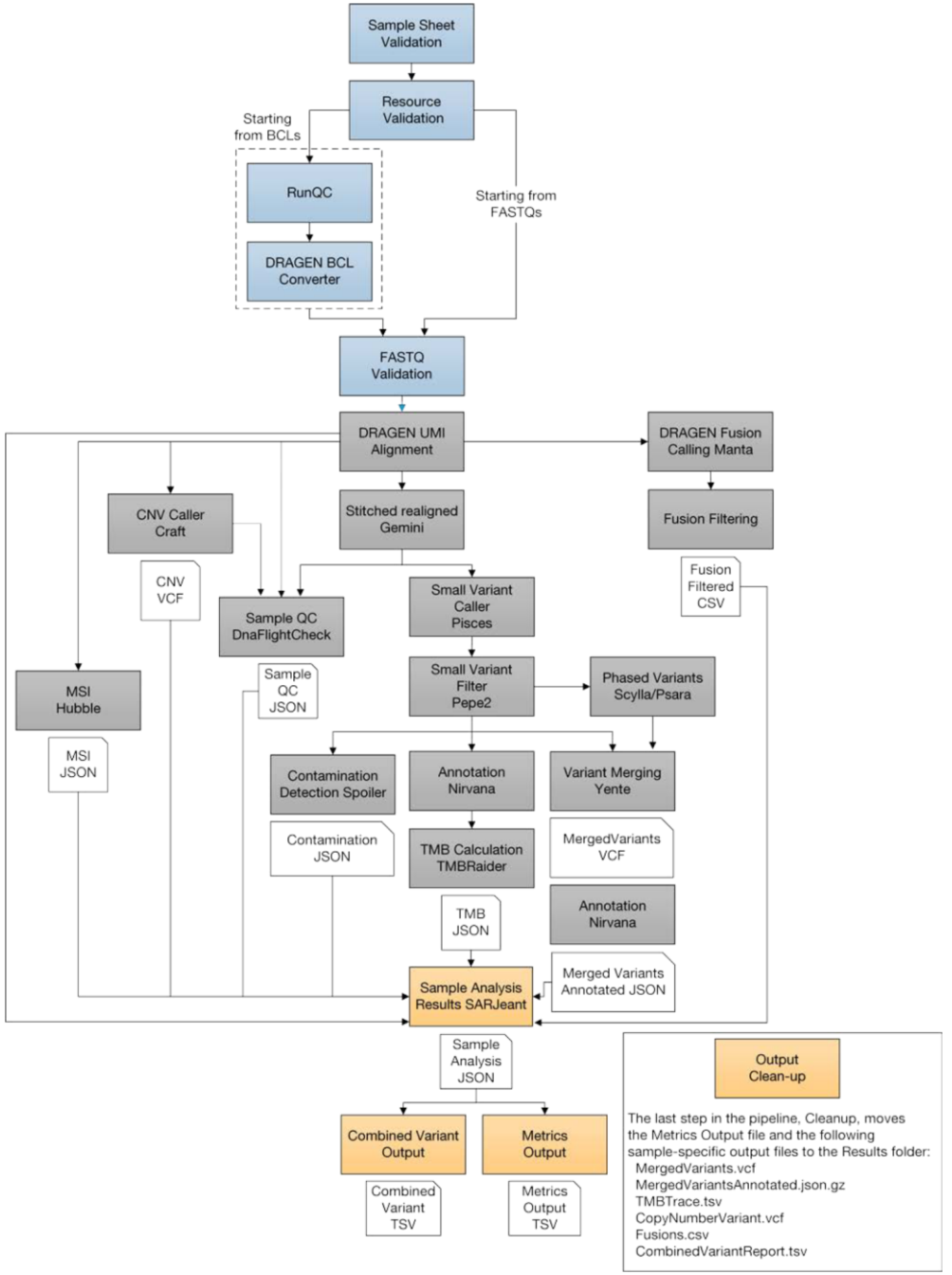
- Panely stovek klinicky relevantních genů
- Standard pro prediktivní testování
- Průkaz alterací na úrovní DNA i RNA
- Stanovení také dalších biomarkerů jako je TMB, MSI, HRD

Pan-cancer: BRAF, NTRK1,NTRK2, NTRK3, RET,MSI, TMB												
	Genes with biomarkers of clinical significance*											Genes with biomarkers of potential clinical significance†
Breast	BRCA1	BRCA2	ERBB2	ESR1	PALB2	PIK3CA						180
Colorectal	ERBB2	KRAS	NRAS									166
Bone	EGFR	ERG	ETV1	ETV4	EWSR1	FEV	FLI1	FUS	H3F3A	HEY1	IDH1	140
	MDM2	NCOA2	SMARCB1									
Lung	ALK	EGFR	ERBB2	KRAS	MET	NUTM1	ROS1					223
Melanoma	KIT	NRAS	ROS1									172
Ovarian	BRCA1	BRCA2	FOXL2									149
CNS‡	APC	ATRX	CDKN2A	CDKN2B	EGFR	H3F3A	HIST1H3B	HIST1H3C	IDH1	IDH2	MYCN	140
	PTCH1	RELA	TERT	TP53								
Prostate	AR	ATM	BARD1	BRCA1	BRCA2	BRIP1	CDK12	CHEK1	CHEK2	FANCL	FGFR2	151
	FGFR3	PALB2	PTEN	RAD51B	RAD51C	RAD51D	RAD54L					
Thyroid	HRAS	KRAS	NRAS	TERT								165
Uterine & cervical	BRCA2	EPC1	ERBB2	ESR1	FOXO1	GREB1	JAZF1	NCOA2	NCOA3	NUTM2A	NUTM2B	138
	PAX3	PAX7	PHF1	POLE	SMARCA4	SUZ12	TP53	YWHAE				
Other solid tumors	ALK	APC	ARID1A	ASPSCR1	ATF1	ATIC	BAP1	BCOR	BRCA1	BRCA2	CAMTA1	152
	CARS	CCNB3	CDK4	CDKN2A	CIC	CITED2	CLTC	COL1A1	COL6A3	CREB1	CREB3L1	
	CREB3L2	CSF1	CTNNB1	DDIT3	DDX3X	DNAJB1	DUX4	EED	EGFR	ERBB2	ERG	
	ETV1	ETV4	ETV6	EWSR1	FEV	FGFR2	FGFR3	FLI1	FOXL2	FOXO1	FOXO4	
	FUS	GLI1	HEY1	HGF	HMGA2	IDH1	KRAS	LEUTX	MAML3	MDM2	MYB	
	MYOD1	NAB2	NCOA2	NF1	NFATC2	NFIB	NR4A3	NRAS	NUTM1	NUTM2A	NUTM2B	
	PALB2	PATZ1	PAX3	PAX7	PDGFB	PDGFRA	PRKACA	PRKD1	RANBP2	ROS1	SDHA	
	SDHB	SDHC	SDHD	SMARCB1	SS18	SSX1	SSX2	SSX4	STAT6	SUZ12	TAF15	
	TCF12	TERT	TFE3	TFEB	TFG	TP53	TPM3	TPM4	TRAF7	TSPAN31	VGLL2	
	WT1	WWTR1	YAP1	YWHAE	ZC3H7B							

TruSight Oncology 500

Datová analýza

- Převod optického signálu do souborů nesoucích informaci o sekvenci a kvalitě čtení této sekvence
- Mapování na lidský genom
- Aplikace nástrojů identifikujících změny oproti referenci – různé nástroje pro jednotlivé typy alterací
- Vytvoření výstupního souboru s výčtem detekovaných změn oproti referenci
- Anotace



DRAGEN TruSight Oncology 500 ctDNA Analysis Software v1.1 User Guide

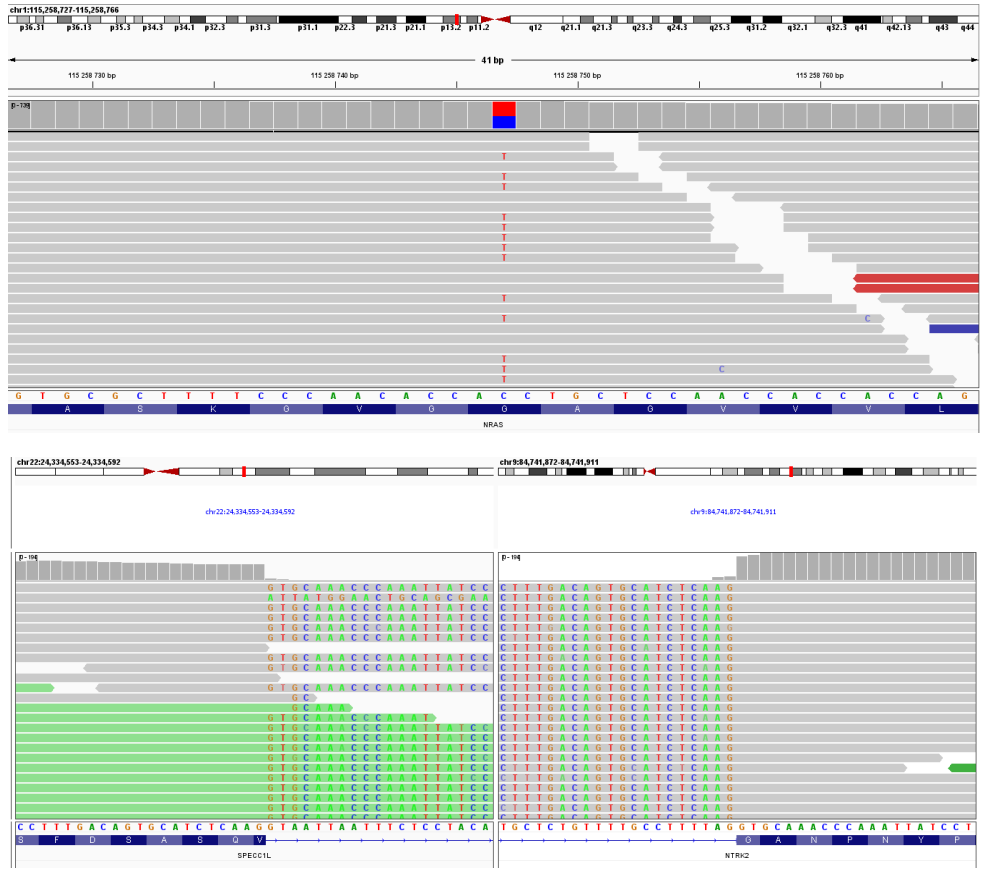
Terciární analýza a interpretace dat

- Filtrování nálezů dle vybraných parametrů (populační frekvence, alelická frekvence, dopad varianty aj.)
- Vizualizace a manuální kontrola detekovaných alterací

Pre-configured Small Variant Demo Filter (29) | Pre-configured TSO 500 RNA Demo Filter (0) | Pre-configured Focal CNV Demo Filter (1) | Pre-configured RNA Demo Filter (2)

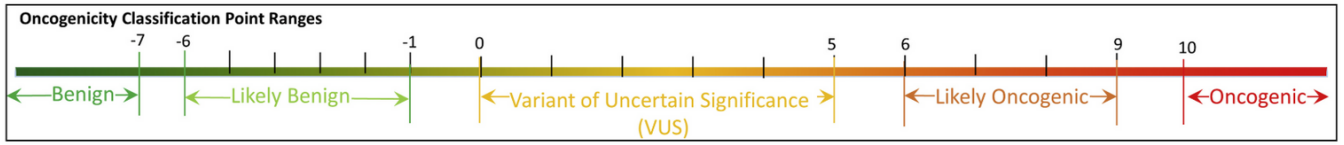
View Variant Filter | Search gene

This Case	Genes	Transcript ...	HGVS / ISCN	VAF	CGC	Consequences	Oncogenicity Prediction
Interpret	PTEN	Exon 7/9	c.640C>T p.(Gln214T...	0.08...	Oncogene, TSG	Loss of Function ... (+ More)	O
Tier 1A O	KRAS	Exon 3/5	c.183A>T p.(Gln61His...	0.1538	Oncogene	Gain of Function V...(+ More)	LO
Interpret	ARID1A	Exon 2...	c.6700del p.(Ala2234...	0.05...	Fusion, TSG	Loss of Function ... (+ More)	LO
Interpret	BAP1 (+1)	Intron 1...	c.2057-4G>T	0.44...	TSG	Loss of Function ... (+ More)	VUS
Interpret	AXIN1	Intron 2...	c.878+8C>T	0.54...	TSG	Loss of Function ... (+ More)	VUS
Interpret	PRKDC	Exon 6...	c.8784G>A p.(Lys29...	0.44...		Loss of Function ... (+ More)	VUS
Interpret	PIK3C2B	Intron 1...	c.2773-9_2773-7deli...	0.9911		Splice Region Vari... (+ More)	VUS
Interpret	AURKB	Exon 9/9	c.885_893delinsTGT...	1		Missense Variant (+ More)	VUS
Interpret	DNMT3A	Exon 3/...	c.89A>C p.(Glu30Ala...	0.502	TSG	Loss of Function ... (+ More)	VUS
Interpret	MLH1 (+1)	Exon 16...	c.1852_1853delinsGC...	0.41...	Fusion, TSG	Missense Variant (+ More)	VUS
Interpret	MLH1 (+1)	Exon 16...	c.1852A>G p.(Lys618...	0.4157	Fusion, TSG	Missense Variant (+ More)	VUS
Interpret	MLH1 (+1)	Exon 16...	c.1853A>C p.(Lys618...	0.41...	Fusion, TSG	Missense Variant (+ More)	VUS
Interpret	PTPRD	Exon 2...	c.2930C>T p.(Thr97...	0.48...		Missense Variant (+ More)	VUS
Interpret	TET1	Exon 4/...	c.3053_3055delinsG...	0.988	Oncogene, Fusion, TSG	Missense Variant	VUS
Interpret	PDCD1	Exon 2/5	c.83C>T p.(Pro28Leu...	0.47...		Missense Variant	LB
Interpret	TENT5C	Exon 2/2	c.895G>A p.(Glu299L...	0.502		Missense Variant	LB
Interpret	NOTCH4	Exon 16...	c.2504G>T p.(Gly83...	0.44...		Missense Variant (+ More)	LB
Interpret	PTPRD	Exon 2...	c.2341A>G p.(Thr781...	0.48...		Missense Variant (+ More)	LB
Interpret	ADGRA2	Exon 12...	c.1621G>A p.(Val541...	0.54		Missense Variant	LB
Interpret	TET1	Exon 4/...	c.3055A>G p.(Lys101...	0.98...	Oncogene, Fusion, TSG	Missense Variant	LB
Interpret	VTCN1	Exon 1/6	c.23T>C p.(Leu8Pro) ...	0.5		Missense Variant (+ More)	LB



Terciární analýza a interpretace dat

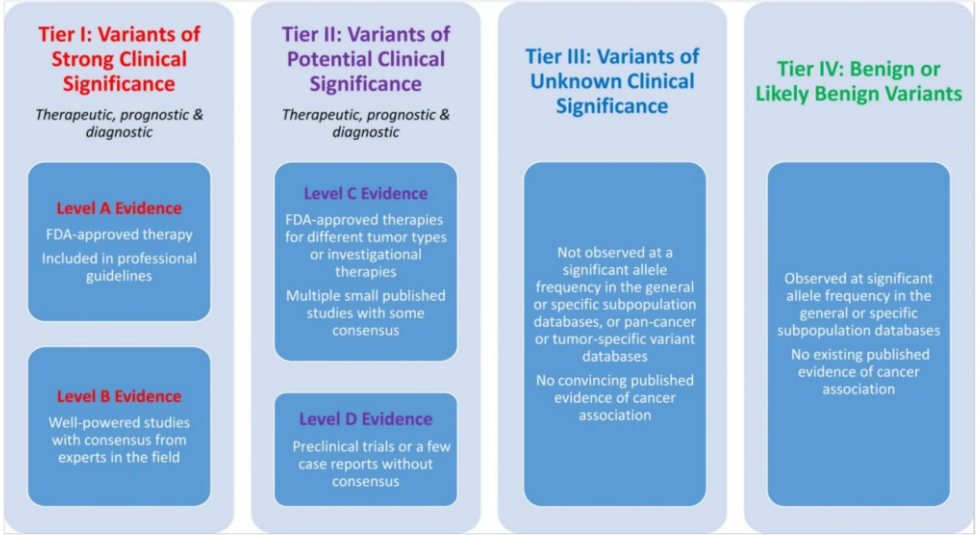
- Posouzení významu detekovaných alterací
- Klasifikace dle mezinárodních schémat
  - Klasifikace patogenicity/onkogenicity
    - ClinGen/CGC/VICC doporučení (PMID: 35101336)

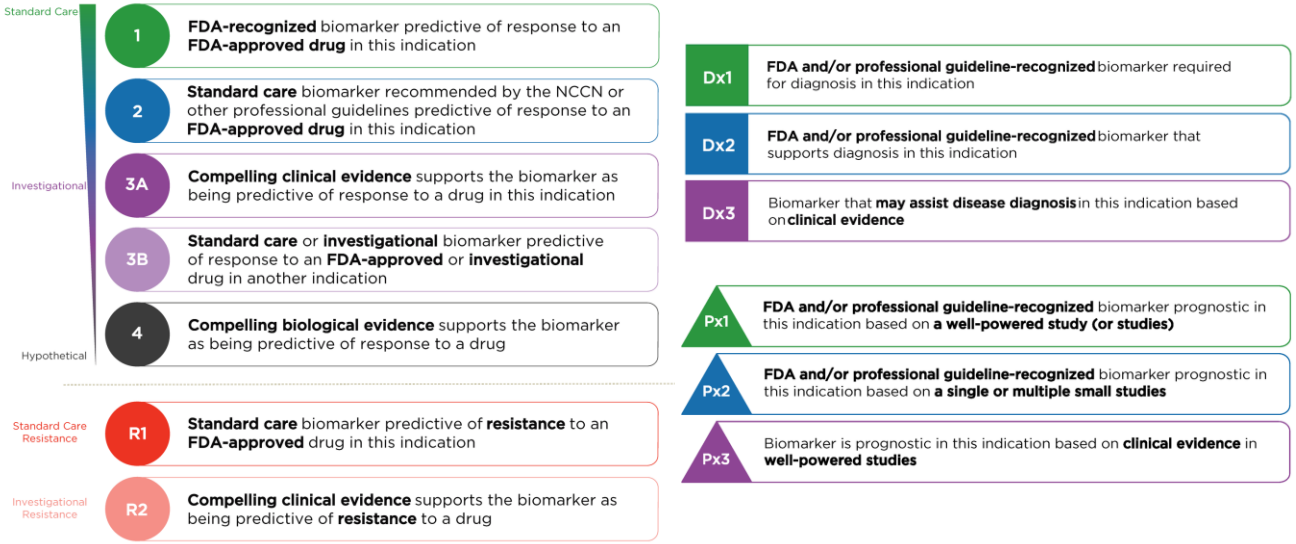


	Benign			Oncogenic			
Evidence Strength	Very Strong	Strong	Supporting	Supporting	Moderate	Strong	Very Strong
POINTS	-8	-4	-1	+1	+2	+4	+8
Population Data	MAF is >5%	MAF is >1%		Absent in population databases			
Functional Data		Well-established functional studies show no oncogenic effects				Well-established functional studies supportive of an oncogenic effect	
Predictive Data			Silent mutation (no predicted impact on splicing)		Missense change at an amino acid residue where a different missense change determined to be oncogenic has been documented	Same amino acid change as a previously established oncogenic mutation	Null variant in tumor suppressor
Cancer Hotspots				Cancer hotspots with low frequency of recurrence	Cancer hotspot with moderate frequency of recurrence	Cancer hotspot with high frequency of recurrence	
Computational Evidence			All utilized lines of computational evidence suggest no impact of a variant	All utilized lines of computational evidence support oncogenicity			

Terciární analýza a interpretace dat

- Klasifikace dle mezinárodních schémat
  - Klasifikace actionability
    - AMP/ASCO/CAP doporučení (PMID: 27993330)
    - OncoKB systém (PMID: 30137196)
    - ESCAT (PMID: 30137196)





Summary

Patient ID: 529893
Tissue ID: T25-17898
Report date: 1-Feb-2024

Patient and sample details

Hospital	University Medical Centre
Patient number	529893 (F)
Date of birth	15-May-1967
Tissue ID	T25-17898
Date of sampling	12-Jan-2024
Sampling anatomical site	Liver
Sample type	Biopsy (FFPE)
Histological cancer type	Adenocarcinoma (NSCLC) (PA-report: T24-12811)
Tumour cell content	Pathology-based: 60% Sequencing-based: 53%
Sample ID(s)	M25-02999, M25-03001
Matched normal DNA	M23-0157
Source of normal	Blood

Assay details

Laboratory	Genomics testing facility
Requested by	Dr. John Doe
Assay description	Comprehensive DNA NGS panel (520 genes), paired tumour-normal analysis, and RNA fusion panel
Assay ID(s)	NGS-DNA-panel01, NGS-RNAfuse-03
Assay run nr(s)	R1565, D9880-1
Report date	1-Feb-2024

Assay quality evaluation

X

PASS, with warnings

Interpretation: The sample is evaluable, but the sensitivity and specificity for gene fusions may be reduced given the relatively low alignment score. Follow-up testing could be considered.

History / Reason for testing

Patient (female) with metastatic NSCLC and a known *ALK* fusion (*EML4-ALK*). Treated with Crizotinib. Sample obtained upon progression after a short initial response. Resistance mechanisms, other targets?

Summary of most relevant findings

Lung adenocarcinoma metastatic biopsy showing:

- *ALK (EML4* ex13 – *ALK* ex20*)* gene fusion. This fusion has also been detected in a previous tumour sample of this patient.

No additional (resistance) mutation are observed. Possible indication for second/third-line ALK inhibitor (e.g. Alectinib, Brigatinib, Lorlatinib, Ceritinib)

- *BRCA1* (p.Ala1708Glu, p.Gln1756fs) inactivation, ESCAT III in lung cancer (possibility for drug repurposing of PARP inhibitors in a clinical trial setting)

- Homologous recombination deficiency (HRD) (score 0.95) confirming the complete (bi-allelic) inactivation of *BRCA1*.

One of the observed *BRCA1* (p.Gln1756fs) mutations is detected in the patient's germline DNA and previously reported as Ashkenazi founder pathogenic variant. Referral to a genetics specialist can be considered.

The combination of two *BRCA1* mutations (bi-allelic) and high a HRD score indicates functional loss of homologous recombination repair function.

To translate these findings into treatment recommendations, further interpretation of the results should be performed by a physician considering the patient's full medical history, if appropriate with support of a molecular tumour board.
An overview of all detected cancer-associated somatic genomic events and germline variants can be found in the report.

The data on which this report is based has been generated using tests that are performed under ISO-17025:2017 TESTING accreditation.
Assay details, sensitivity/specificity specs and clinical validation reports are available via genomics-facility.org/ISO-val-reports/.

Based on a minimal tumour purity of at least 20%, the test has a sensitivity of >95% for detection of genomics variants, >95% for copy number alteration and >95% for detection of gene fusions.

Genes/regions that are covered by the assay(s) design and/or that are included in the clinical reporting can be downloaded here: www.genomics-facility.org/assays/. Further details about the bioinformatics pipeline and analysis parameters can be found on GitHub: (https://github.com/seqfacility/)

This report has been generated on 1-Feb-2025 using the pipeline version 2.1. A user manual to assist in interpretation of this report can be downloaded here: www.genomicsfacility.org/test-manuals/.

For questions, feedback or complaints please contact support@genomics-facility.org

Results

Patient ID: 529893
Tissue ID: T25-17898
Report date: 1-Feb-2024

Mutations

Assay/analysis QC: X

Gene	Type	Transcript	Position	Variant	Effect	Reads	VAF	LoH	Germline	Hotspot	Class
TP53	TSG	NM_000546.6	17:7577124	c.814G>A (p.Val272Met)	missense	372 / 610	61%	yes	no	yes	Likely pathogenic
BRCA1	TSG	NM_007294.4	17:41209079	c.5266dup (p.Gln1756fs)	frameshift	297 / 571	53%	no	yes	yes	Pathogenic
BRCA1	TSG	NM_007294.4	17:41215920	c.5123C>A (p.Ala1708Glu	missense	231 / 662	35%	no	no	yes	Pathogenic
TERT	ONCO TSG	NM_198253.3	5:1295228	c.-124C>T	promoter	238 / 366	65%	no	no	yes	Pathogenic
KEAP1	TSG	NM_203500.2	19:10602583	c.996del (p.Gly332fs)	frameshift	287 / 521	55%	yes	no	no	Likely pathogenic
KDM6A	ONCO TSG	NM_001291415.2	X:44922943	c.1804G>A (p.Gly602Arg)	missense	199 / 398	51%	no	no	no	VUS

Copy number alterations

Assay/analysis QC: X

Gene	Type	Transcript	Region	Event	Gene copy number	Chr arm copies
RB1	TSG	NM_000321.3	13q14.2	loss	0	4
NCOA2	ONCO	NM_006540.4	8q13.3	gain	10	2

Gene fusions

Assay/analysis QC: X
Comment: Warning, low alignment score*

Gene 5'	Transcript 5'	Exon 5'	Gene 3'	Transcript 3'	Exon 3'	Phasing	Reads
EML4	NM_019063.5	13	***ALK***	NM_004304.5	20	in-frame	135/211 (88%)
HEY1	NM_012258.4	3	***NCOA2***	NM_006540.4	10	exon-skipping	6/14 (43%)

**Gene fusions show a relatively low number of aligned reads, indicating a lower sensitivity/specificity, likely due to lower quality of the input tissue. Caution is required for interpretation of the results*

Mutational signatures

Assay/analysis QC: X

Signature	Score	Threshold [range]	Result
Tumour mutation burden (TMB)	2.4	10 [0-100+]	Low
Microsatellite (in)stability	0.1	4 [0-100+]	MSS (stable)
Homologous recombination deficiency	0.95	0.5 [0-1]	Deficient

Genomics aberrations are reported based on the HG37 genome assembly and the MANE select transcript IDs (or the transcript indicated) (last update 01-Jan-2023). For reported (>3variants, a loss-of-heterozygosity status has been determined to indicate complete bi-allelic loss of the wildtype allele. Variants are classified as (likely) pathogenic, variant of unknown significance (VUS). (Likely) benign variants are not included in this report. Variants that are (likely) present in the patient's germline are indicated. Genes are marked as Oncogenes (ONCO) and/or tumour suppressor genes (TSG) and variants in known hotspots codons are indicated. (source: Jackson CKB, as per 1-Jan-2024). More details and an overview of the genes that are considered for variant reporting can be found here: www.genomics-facility.org/assays/variant-reporting/

Copy number alterations are reported for copy gains x chromosomal arm copies). For copy losses, only events are reported that show a complete loss (0 copies). Gene losses are reported for TSG only, gene gains (amplifications) are called for ONCO. More details and an overview of the genes that are considered for fusion reporting can be found here: www.genomics-facility.org/assays/CNV-reporting/

Gene fusion detection has been performed using RNA-based sequencing. Fused gene products are reported in case of in-frame events, including in-frame events that require exon skipping. Gene fusions are preferentially detected on the MANE select transcripts but can also include alternative transcripts. The number and percentage of fusion reads are reported. More details and an overview of the genes that are considered for fusion reporting can be found here: www.genomics-facility.org/assays/fusion-reporting/

Reported genomic signatures include the tumour mutational burden (TMB) as number of missense muts/Mb, microsatellite instability (MSI), and homologues recombination deficiency (HRD). Details of the used procedure and bioinformatics pipeline can be found on GitHub (https://github.com/seqfacility/)

Biomarkers clinical evidence

Patient ID: 529893
Tissue ID: T25-17898
Report date: 1-Feb-2024

Clinical actionability annotation

Gene/Biomarker	Alteration	Match level	Treatment type/ drug	ESCAT	Source
ALK	***ALK-EML4 fusion***	fusion	Alectinib, Lorlatinib, Crizotinib, Ceritinib, Brigatinib	I	CKB-JAX
ALK	***ALK-EML4 fusion***	fusion	Alectinib, Lorlatinib, Crizotinib, Ceritinib, Brigatinib	I	OncoKB
BRCA1	**p.Ala1708Glu, p.Gln1756fs**	inactivation	Olaparib, Talazoparib, Niraparib	III	CKB-JAX
BRCA1	**p.Ala1708Glu, p.Gln1756fs**	inactivation	Rucaparib	III	CKB-JAX
BRCA1	**p.Ala1708Glu, p.Gln1756fs**	oncogenic	Olaparib, Niraparib, Rucaparib	III	OncoKB
HRD	**Positive**	signature	PARP inhibitors	III	CKB-JAX

Clinical trial biomarker matching

Gene/Biomarker	Alteration	Match level	Trial name/number	Phase	Source
BRCA1	p.Gln1756fs	inactivation	NCT04826341 NCT03845296 NCT05700721	I/II II II	CKB-JAX
HRD	Positive	signature	NCT04826341 NCT05700721	I/II II	CKB-JAX

Findings for potential follow-up

Gene/Biomarker	Alteration	Follow-up	Remark
BRCA1	c.5266dup (p.Gln1756fs)	Referral to genetic counselling/cancer genetics specialist	Pathogenic variant also observed in the patient germline DNA. Reported as Ashkenazi founder pathogenic variant.
HEY1::NCOA1	*HEY1* ex3 – *NCOA1* ex10	Confirmatory testing is required if considered for the pathological diagnosis.	Low RNA reads, potential passenger due to *NCOA2* amp.

Reported genomic events are matched against knowledgebases (source: Jackson CKB, OncoKb, as per 1-Jan-2024) for associated clinical evidence regarding potential treatment approaches. ESCAT levels are used for clinical relevance; only evidence items with an ESCAT tier I, II or III are reported. Clinical evidence matching is performed for (likely) pathogenic variants (VUS are not included), copy numbers alterations, fusions and genomics signatures. Only the evidence items with the highest tier match are shown.

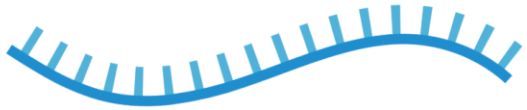
Clinical trial matching is performed based on the genomics event and tumour type information only. Other clinical trial eligibility criteria are not considered, and need to be evaluated further if inclusion in a clinical trial is considered.

PMID: 39112111

Shrnutí

DNA

RNA



Protein

	SNV/InDels	SVs	Del/Amp	MSI	TMB	HRD
FISH	✗	✓	✓	✗	✗	✗
PCR	✓	✓	✗	✓	✗	✗
NGS	✓	✓	✓	✓	✓	✓

	SNV/InDels	SVs	Del/Amp	MSI	TMB	HRD
PCR (RT-PCR)	✗	✓	✗	✗	✗	✗
NGS	✗	✓	✗	✗	✗	✗

	SNV/InDels	SVs	Del/Amp	MSI	TMB	HRD
IHC	✓*	✓*	✓*	✓	✗	✗

*omezené spektrum

*vybrané, nepřímo

*nepřímo, pouze amplifikace

Shrnutí

	Výhody	Nevýhody
IHC	Levná, rychlá a dostupná metoda Hodnocení na úrovni jednotlivých buněk	Inter-observační variabilita Specificita a dostupnost protilátek
FISH	Vysoká přesnost Hodnocení na úrovni jednotlivých buněk	Omezený počet cílů v 1 reakci Vyšší náročnost na vybavení
PCR	Relativně levná a rychlá metoda Jednoduchá interpretace	Omezený počet cílů v 1 reakci
NGS	Komplexní metoda umožňující detekovat široké spektrum alterací	Vyšší cena a náročnost na vybavení Složitost datové analýzy a interpretace